EXHIBIT A

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Applications of Fluorescence in Immunoassays

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PREFACE

Fluorescence provides a diversified and sensitive detection system applied in the versatile field of immunological techniques. The application of antibodies labeled with fluorescent probes dates back to the 1940s, when Coons et al. introduced the microscopic immunofluorescence staining technique. During the 1970s fluorescence was considered as a promising and potentially very sensitive detection system in the search for alternative labels to replace radioisotopic tracers in immunoassays. Regardless of the number of assays developed and also successfully applied in certain areas, the inherent vulnerability of fluorescence detection to background interferences hindered its application in assays requiring high sensitivity. The recent development of fluorescence instruments, assay technologies and fluorescent probes has, however, resulted in assay techniques producing one of the highest available sensitivities, and fluorometric immunoassays also offer real alternatives to the sensitive radioisotopic immunoassays. The ability of fluorometric detection to combine spectral, temporal and spacial resolution offers a powerful tool for future immunoassay development, too.

The present monograph describes the basic prerequisites for a fluorometric immunoassay; the antibody, the immunological technology, the fluorescent probe and the instrument, as well as gives a profile of the clinical applications of the various assay technologies.

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the inherent nitrogen as a marker. The use of fluorescent compounds as sensitive marker substances coupled to antibodies was invented by Albert Coons and his colleagues in the early '40s, when they developed immunofluorescence staining techniques for microbes (5, 6).

The study of antibody production in diabetic patients treated with insulin led to the development of the radioimmunoassay in the late '50s by Berson and Yalow (7, 8); this method has had a major impact on the acceptance of immunological techniques in the field of routine clinical diagnosis.

In their early days radioimmunoassays were exclusively applied for determinations of peptide hormones. Since the pioneering work of Landsteiner in 1946 (9), antibodies have also been produced for small (molecular weight under 10,000) compounds called haptens, for compounds which as such are unable to elicit antibody production but must be bound to larger carrier molecules to form immunogenic conjugate. The production of antisera against haptenic molecules, such as steroids (10) or thyroid hormones (11), opened a new dimension for immunoassays. Since then antibodies have been produced against an enormous number of antigens and biological and synthetic compounds, and these have been applied in a variety of ways for analyzing those compounds. Modern biotechnology has revolutionized antibody production, and genetic engineering opens totally new perspectives for their future applications.

2.1. IMMUNOGENIC RESPONSE

An antigen is an immunogenic compound which can elicit a strong immune response in an immunized animal. An immunogenic antigen can be a peptide, protein, polysaccharide, polynucleotide, or almost any polymeric compound containing functional groups on its surface recognized by antibody producing B-lymphocytes. The primary recognition by the membrane bound receptor proteins of lymphocytes triggers the complex process of maturation of antibody producing B-cells and the subsequent production of large quantities of antibodies.

The production of antisera of high titer, affinity, and specificity requires substantial amounts of chemically pure antigens. A large amount is needed for repeated immunizations of test animals. High purity is an absolute necessity in order to obviate cross-reactivities with unrelated compounds. The purification and stability problems with some biological compounds can be a limiting factor in antiserum production, but these have been partly overcome with the development of methods for producing monoclonal antibodies (Chapter 2.2).

2.1.1. Haptenic Antigens

Haptenic antigens are compounds which because of their small size cannot elicit immunoresponse. Generally the molecular weight limit for immunogenic response is around 10,000. Because of the difficulties in producing anti-hapten antibodies, the first real immunoassays were developed for peptides or proteins, and actually the first "specific protein binding assays" of haptenic molecules, developed by Roger Ekins et al. in the early '60s, use naturally occurring specific binding proteins, thyroxine binding globulin for labeled thyroxine (12) and intrinsic factor for labeled B_{12}-vitamin (13).

The production of anti-hapten antibodies was invented in the late '40s (9), and anti-steroid antibodies were produced in 1957 (10). It was several years, however, before these were applied for making radioimmunoassays. For eliciting immunoresponse the haptenic molecules need first to be coupled to a suitable carrier. Bovine serum albumin is the most often used carrier protein for immunizations, mainly because of its solubility and availability. Other proteins, like keyhole limpet hemocyanin, have been preferred later on because of their high immunogenicity and coincident contribution of the production of anti-hapten antibodies with high titer and affinity (14).

The production of anti-hapten antibodies of predetermined specificity is often problematic, partly because the coupling of the compound to a carrier can block important epitopic sites needed for specificity and partly because of recognition of the linking arm between the hapten and carrier by the produced antibodies. Since the antibodies are able to bind structures equal to about 7 amino acid residues (15), an anti-hapten antibody most often recognizes simultaneously part of the linking group and spacer arm used in conjugation reaction for immunization (bridge recognition).

Bridge recognition is especially problematic for steroid immunoassays (16, 17) and is encountered when labeled steroids (tracer) or immobilized steroids (e.g., solid-phase reagent) are prepared using the same position of the steroid (site homology) or same linking arm (bridge homology) as used for preparing the immunogenic conjugate. With such conjugates the competitive binding between the limited amount of antibody, labeled antigen, and the unknown amount of sample antigen (or standard) favors the reaction between tracer and antibody with poor replacement; the rate constant k_t is much higher than k_s (Eq. 2.1). The poor replacement results in insufficient slope to the standard curve and low assay sensitivity because the sample antigen is unable to compete with the tracer in binding to antibodies.

Accordingly, the production of immunogenic conjugates for steroid immunization is better performed after selecting different spacer arms or sometimes even different positions for attachment on the steroid structure (Fig. 2.1).

$$\mathrm{Ab} \begin{array}{l} + \mathrm{Ag-F} \underset{}{\overset{k_1}{\rightleftharpoons}} \mathrm{Ab-Ag-F} \\ + \mathrm{Ag} \underset{}{\overset{k_2}{\rightleftharpoons}} \mathrm{Ab-Ag} \end{array} \tag{2.1}$$

For example, considerably higher sensitivity was obtained in an assay of 17-hydroxyprogesterone when using a bridge heterologous tracer as compared to a respective homologous system (18). Similarly, the equilibrium time required for ligand displacement in an assay of estradiol shortened from 10 h to 1 min when changing from a homologous system to heterologous (19). The requirement of site homology depends greatly on the analyte and antibodies used. In BIA of cortisol Arakawa et al. (20) used cortisol-6α-hemisuccinate for producing the antigen conjugate for solid-phase immobilization and 3-carboxymethyloxime conjugate for producing the marker-enzyme tracer. On the other hand, in the experiments of Kobayashi et al. (21) and Mikola and Miettinen (22), cortisol could be assayed only with a site homologous system. Tielemauer and Andres (23) tested spacer arms between estradiol and biotin for use in BIA. They found that a reasonably long spacer was an absolute necessity and that the chemical structure of the spacer may also have a major effect on bridge recognition.

2.2. MONOCLONAL ANTIBODIES

In 1975 Köhler and Milstein (24) made the first monoclonal antibodies of predetermined specificity by fusing a spleen cell line producing the specific antibodies with a myeloma cell line capable of continuous growth in cell culture. Since then the advent of monoclonal antibodies has had an enormous impact on many fields of biomedical research (25, 26). It was soon realized that the technique would revolutionize the immunoassay field as well, and it has raised great expectations also in immunotherapy, imaging, and biotechnology.

Monoclonal antibodies are rapidly gaining a dominant position in immunoassays, especially from a commercial point of view, because of their unlimited supply, molecular homogeneity, and defined, unchanged properties. The production and use of monoclonal antibodies has also become a

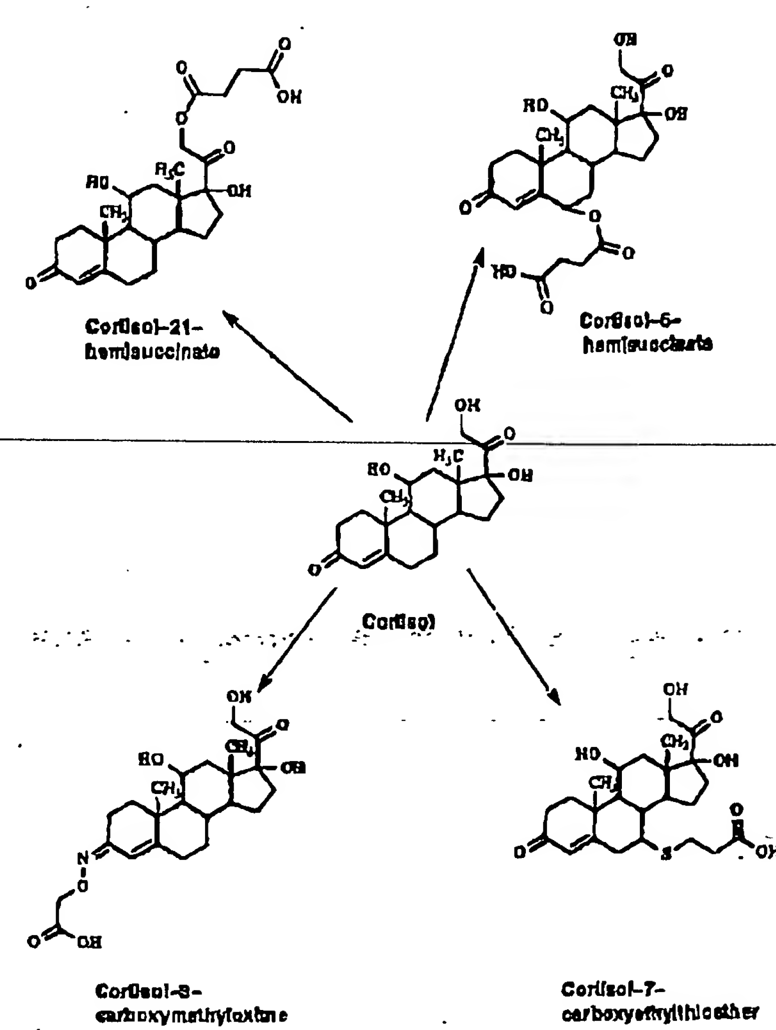

Fig. 2.1. Site heterologous routes to prepare cortisol derivatives for immunization and for labeling.

and it was about 20 years before FPIA reached routine clinical laboratories—when Abbott adopted the technology and developed clinical instruments and numerous kits based on FPIA technology.

Today numerous homogeneous assay principles have been introduced. Several technologies are commercialized and have found quite extensive applications in certain areas, especially in measuring drugs (therapeutic drug monitoring, TDM, and tests for illicit drugs).

8.3.1. Fluorescence Polarization Immunoassays

The efficiency of light absorption by a fluorophore is dependent on the angle between the electronic dipole of the exciting light and the absorption oscillators of the molecule. A polarized light will excite only those molecules that have their absorption oscillators parallel to the plane of exciting light. The polarization level of the resultant emission depends on the lifetime of the excited state (τ) and the rotational motion of the molecule. For steady-state measurement polarization is generally expressed by the Perrin equation (Eq. 8.1) (1182).

$$(1/p - 1/3) = (1/p_o - 1/3)(1 + 3\tau/\rho) \tag{8.1}$$

The rotational relaxation time, ρ, can be calculated for a spherical molecule according to Equation 8.2.

$$\rho = 3\,\eta\, v/kT \tag{8.2}$$

Rotational relaxation time is directly proportional to the volume of the molecule (size and shape) and viscosity (η) of the medium.

A large molecule, such as an antibody, has a tumbling time typically around 10 to 100 ns, whereas small molecules, such as haptens, have tumbling times around 0.1 to 1 ns. In steady-state polarization measurement (continuous excitation with polarized light), the resulting polarization of emission depends on the size and shape of the labeled substance and the ratio of rotational relaxation time to the decay time of the fluorochrome. This forms the basis for measuring binary binding reactions—for example, in immunoreaction.

To be practical for an immunoassay, the change in molecular volumes during the immunoreaction needs to be high enough, such as it is during the binding of the haptenic tracer to its antibodies. It gives a practical limit for the size of antigen, which should be below 20,000. The decay time of the fluorophore needs to be longer than the rotational time of the haptenic tracer but shorter than the rotational time of the formed complex. Fluorescein (τ

4.5 ns) accordingly works very well for normal FPIA, the polarization of which increases drastically upon the binding of the fluorescein-labeled hapten to the respective antibodies.

For large antigens, fluorochromes with somewhat longer decay times have been tested. With proteins the intramolecular tumbling becomes problematic, however, when using steady-state measuring (1183). So far no applications have been made using large binding entities (e.g., microbeads) and long τ probes.

In addition to the size limitations of FPIA, problems arise also from the low affinity nonspecific binding properties of serum proteins, especially that of albumin, which increases the polarization level nonspecifically. To avoid the albumin effect, a simple dilution jump has been used (1184), or various sample pretreatments are required. In the pretreatment solutions either chaotropic ions, proteolytic enzymes, protein precipitating reagents, or solvents are used. The pretreatment of samples is especially needed for analytes that require a high sensitivity that does not allow for high dilutions.

The principle of fluorescence polarization was developed by Perrin in 1926 (1182). About 30 years later, in 1952, the technology was applied in biological systems by Weber (1185). For monitoring immunoreactions, the fluorescence polarization technique has been used since 1961, since the pioneering work of Dandliker et al. (277), who studied the interaction of fluorescein-labeled penicillin (279), ovalbumin (280), and estrone (1186) with their specific binding proteins or receptors. Dandliker has also written a number of review articles about the principle and applications of fluorescence polarization (282, 1184, 1187).

The experimental studies of FPIA during the 1960s and 1970s were conducted with research fluorometers equipped with polarization accessories and have resulted in a limited number of clinical applications (Table 8.9), mainly because of the lack of appropriate instruments for routine assays.

Table 8.9. Early Applications of Fluorescence Polarization in Protein Binding Assays

Analyte	Tracer	Assay type	Reference
Anti-penicillin-Ab	FITC-Penicillin	Direct	279
Estrone receptor	FITC-Estrone	Direct	1186
Anti-ovalbumin-Ab	FITC-Ovalbumin	Direct	280
Anti-Conalbumin-Ab	FITC-Conalbumin	Direct	281
Trypsin	FITC-Casein	Direct	283
Anti-insulin-Ab	FITC-Insulin	Direct	283
hCG	FITC-hCG	Competitive	282, 1188
Fungicides	FITC-Ag-s	Competitive	1189

Table 8.10. Applications of TD_x

	Analyte	Reference	Evaluation
Antibiotics			
	Gentamicin	1192	1193–1196
	Tobramycin	1192	
	Amikacin	1192	1197
	Streptomycin	1198	
	Netilmicin		1195, 1199
	Isepamicin		1200
	Vancomycin		1201
	Astromicin		1202
Anticonvulsants			
	Phenytoin	365, 1203	1194, 1204, 1205
	Phenobarbital	365, 1203	1194, 1204
	Carbamazepine		1204, 1206, 1207
	Valproic acid		1204, 1208
Antiarrhythmics			
	Quinidine/free		1209, 1210
	Hydroquinidine		1211
	Lidocaine		1212
	Disopyramide/free	1213	
	MEGX	1214	
Other drugs			
	Theophylline	1215, 1216	1194, 1217, 1218
	Digoxin		1194, 1219–1221
	Methyldigoxin	1222	
	Benzodiazepine	1223	1224, 1225
	Flecainamide		1226
	Paracetamol		1227
	Methotrexate		1228
	Cyclosporine	1229–1231	1232–1243
Illicit drugs			
	Morphine	1244	
	Amphetamine	1245	1246
	MHPG	560	
	Barbiturates		1247
	Opiates		1247
	Cocaine metab.	1248	1249–1251
Hormones			
	Thyroxine (T_4)	1252	1253, 1254
	FT_4		1253
	T_3	1255	
	T uptake	1256	
	Cortisol		1257
Hormones			
	Free cortisol/U		1258
	Estriol	1259	
	OH-indole acetate	1260	
Proteins and peptides			
	Angiotensin	1261	
	CRP	1262	1263, 1264
	Transferrin		1265

Table 8.10. Applications of TD_x *(continued)*

Wider usage of competitive FPIA in clinical routine started during the early 1980s, when Abbott introduced an automated instrument designed for clinical FPIA applications (455, 456, 1190). With instruments of various stages of automation (TD_x, AD_x and IM_x) and over 30 different kits, FPIA has become one of the most used FIA in clinical chemistry (379, 1191). Lately Abbott has been accompanied by other reagent and instrument manufacturers, such as Roche Diagnostics, CANAM Diagnostics, Colony, Sankyo, Innotron of Oregon Inc. (INNOFLUOR™ FPIA), Polymed Co. and Source Scientific Systems (Focus™ FPIA fluorometer).

The reagent pack of TD_x generally contains a pretreatment solution, antiserum, and antigen labeled with a fluorescein derivative. The instrument performs the required dilutions, records the blank value to be substracted, and measures the final polarization level. The technology is used primarily in TDM and screening for illicit drugs, but it is also used for some hormones and even for a few proteins, such as globulins, transferrin, and CRP. Table 8.10 summarizes examples of the articles describing FPIA applications performed on TD_x, AD_x, or the automated IM_x, including the numerous evaluations of the existing kits and other FPIA applications of the TD_x instrument. Some of the assays, such as the assay of cyclosporine, have sparked a great number of evaluations, partly collected in the table.

The research group of Prof. Landon has developed FPIAs since 1976 (1266) and has developed analytical applications for the determination of hormones and drugs. They have been able to simplify the technology further by using a one-step, one-reagent method based on antibodies pre-equilibrated with FITC-labeled antigens. By adjusting the respective affinities so that ligand displacement can take place rapidly, this LIDIA principle provides an extremely simple and rapid analysis. Assays are performed with various research fluorometers, including the Perkin Elmer LS 20 Polarisation Fluorometer particularly developed for clinical routine assays (457). FPIA applications performed with homemade reagents are listed in Table 8.11

Table 8.11. Research Applications of FPIA

	Analyte	Label	Reference
TDM			
	Gentamicin	FITC	1266
	Phenytoin	FITC	372
	Phenytoin	2-Naphthol-sulfonamide	390
	Valproic acid	FITC	1267
	Paracetamol	FITC	487, 1268
	Quinine	FITC	1269
	Theophylline	FITC	489
	Theophylline	Umbelliferyl	672
	Salicylate	FITC	1270
Illicit drugs			
	Opiates	FITC	345
	Amphetamine	FITC	486, 1271
	Methamphetamine	FITC	344, 488
	Benzoylecgonine	FITC	490
	Barbiturates	FITC	1272–1274
	Vanillylmandelate	FITC	1275
	Azidothymidine	FITC	1276
Hormones			
	Cortisol	FITC	370, 373, 1277
	Biopterin	FITC	1278
	Neopterin	FITC	1278
	Deoxycortisol	FITC	1279
	Estradiol	Fluorescein	19
	Estriol	Lucifer Yellow	688
	Testosterone	Lucifer Yellow	688

New manufacturers producing FPIA kits have recently emerged. The kits are intended to be measured either with the existing Abbott TD_x system or with the manufacturer's own instrument, such as the Roche FPIA, developed for the company's Cobas Bio and Cobas Fara chemistry automates. At the moment, alternative products are concentrated in drug monitoring (Table 8.12).

Relatively little effort has been used to develop FPIAs for larger molecules such as proteins. The problems with proteins are related to their large size and their flexibility, as well as the lower sensitivity and more narrow dynamic range obtainable. TD_x has, however, been applied to some proteins, such as globulins, ferritin, and CRP (Table 8.10) and to analytes that do not require high sensitivities.

Table 8.12. Alternative Commercial FPIA Assays

Analyte	Company	Reference
Gentamicin	Roche Diag.	1280
	IBC, Innotron Diag.	1281
Tobramycin	Roche Diag.	1280
	IBC, Innotron Diag.	1281
Vancomycin	Roche Diag.	565
Phenytoin	Roche Diag.	566, 1282
	IBC, Innotron Diag.	1281
Phenobarbital	Roche Diag.	566
	IBC, Innotron Diag.	1281
Carbamazepine	Roche Diag.	566
Theophylline	Roche Diag.	1280, 1283
	CANAM	1281
	IBC, Innotron Diag.	1281
	Colony	1284
Quinidine	Roche Diag.	566, 1282, 1285
Primidone	Roche Diag.	1286
Digoxin	Roche Diag.	1287
Procainamide	Roche Diag.	566, 1282
NAPA	Roche Diag.	566
Dilantin	Roche Diag.	1280

Urios et al. (1188) have made an FPIA for urine hCG using FITC-labeled hCG as the tracer. Reportedly they observed a rather wide dynamic range in the assay—from 0.27 to 64 µg/ml. Yamaguchi et al. (501) were able to measure insulin with a competitive FPIA with a dynamic range from 40 to 600 mU/ml, but only from pure insulin preparations and not from serum samples. A similar insulin FPIA has also been tested by Nithipatikom and McGown (1288), who studied the fluorescence intensity changes, decay-time changes, and polarization changes of FITC-labeled insulin during the immunoreaction. Assays of smaller peptides, such as angiotensin (1261) and neocarzinostatin (454), can be developed more easily.

One approach toward protein FPIAs has been the development of fluorescent probes with longer decay times. Dansyl and umbelliferone derivatives have been tested for FPIA of CK-MB (617). The longer decay-time emitter, dansyl derivative, proved to be too flexible to give an acceptable polarization level. Urios and Cittanova used Lucifer Yellow as a label in a direct assay of IgM (1289). The assay was based on a smaller binding unit, the Fab-fragment of a monoclonal antibody, labeled with the fluorophore and used as a direct reagent for the larger antigen, IgM.